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Isolation of an Actin Polymerization Stimulator from Bovine Thyroid Plasma Membranes

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An actin polymerization stimulator was purified from bovine thyroid plasma membranes by DNase I affinity column chromatography. Although the molecular weight of the protein was about 42,000 (42K) by sodium dodecyl sulfate polyacrylamide gel electrophoresis, it did not comigrate with actin. In the presence of 30 mM KCl, the 42K protein facilitated formation of actin filaments when analyzed by a centrifugation method, accelerated the initial phase of actin polymerization as measured in an Ostwald viscometer and increased the length of filaments as shown by electron microscopy. The 42K protein also accelerated the initial phase of actin polymerization in the presence of 100 mM KCl and 2 mM $MgCl_2$ but did not affect the final viscosity. The effect of the 42K protein was diminished by 5 uM cytochalasin B or 1 uM cytochalasin D. This 42K protein may anchor actin filaments onto the thyroid plasma membrane.

Among endocrine glands, the thyroid is unique in that it transports the prohormone thyroglobulin to the follicular lumen by exocytosis, stores it in the colloid (1) and then produces thyroid hormones by endocytosis (2) and subsequent proteolysis of colloid. Morphological studies demonstrate abundant microfilaments in microvilli and pseudopods in the apical membrane area of thyroid follicular cells (3,4) and association of actin and myosin with thyroid plasma membranes was demonstrated (5). These contractile proteins have been implicated in exocytotic and endocytotic processes involved in thyroid hormone synthesis and secretion (6,7). In contrast to the relatively permanent structure of actin filaments in skeletal muscle, non-muscle cells have a system to control the

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transient assembly and disassembly of microfilaments (8-15). Thus, profilin (8,9), a low molecular weight protein, and nucleator proteins such as gelsolin (10), villin (11-13), fragmin (14) and 90K protein from human platelets (15) have been identified. We recently purified, from thyroid gland, Ca^{2+}-dependent actin modulator proteins which inhibited skeletal muscle actin polymerization in the presence of calcium (16). We now report isolation from bovine thyroid plasma membranes of another protein which facilitated skeletal muscle actin polymerization.

Materials and Methods

Pancreatic DNase I, cyanogen bromide activated Sepharose 4B, Coomassie Brilliant Blue G-250, cytochalasin B, cytochalasin D, phenylmethylsulfonyl fluoride, antipain and leupeptin were obtained from Sigma Chemical Company. Acrylamide, bis acrylamide, sodium dodecyl sulfate, N,N,N',N'-tetramethylethylenediamine, ammonium persulfate and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (17). Bovine plasma membranes were purified by the method of Yamashita and Field (18). Skeletal muscle actin was purified according to the method of Spudich and Watt (20). Pancreatic DNase I was covalently coupled to cyanogen bromide activated Sepharose 4B as reported by Lazarides et al (21). Protein concentration was determined by the method of Lowry et al (22) or by the method described by Bradford (23) using γ-globulin as a standard.

Actin polymerization was initiated by addition of various concentrations of KCl or $MgCl_2$ in G buffer (3 mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$, 0.5 mM ATP, 6 mM 2-mercaptoethanol and 0.02% NaN_3) at 25°C. Effects of the 42K protein on skeletal muscle actin polymerization were analyzed as follows. In the centrifugation method (24), skeletal muscle actin was incubated at 25°C for 15 min with or without 42K protein under various conditions as indicated in the text. The samples were then centrifuged at 100,000 x g for 60 min and the supernatants analyzed by SDS-gel electrophoresis. The time course of polymerization of skeletal muscle actin under various conditions was measured based on viscosity changes and high shear rates in a Cannon-Manning semi-micro viscometer (extra low charge; size 100). The buffer flow time was between 52 and 54 seconds at 25°C. Specific viscosity was defined as sample flow time divided by buffer flow time minus 1. Polymerized actin solution was placed on a carbon and Formovar-coated microscopic grid and negatively stained with 1% uranylacetate and examined with a Phillips electron microscope.

Results

Except for DNase I affinity column chromatography, which was done at room temperature, the procedure for purifying the stimulator of actin polymerization was carried out at 4°C. Bovine thyroid plasma membranes were solubilized with 1% Triton X-100 in 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$ and 0.85% NaCl. After centrifugation at 30,000 x g for 20 min, the supernatant solution was dialyzed overnight against two changes of G buffer. The dialyzed supernatant solution

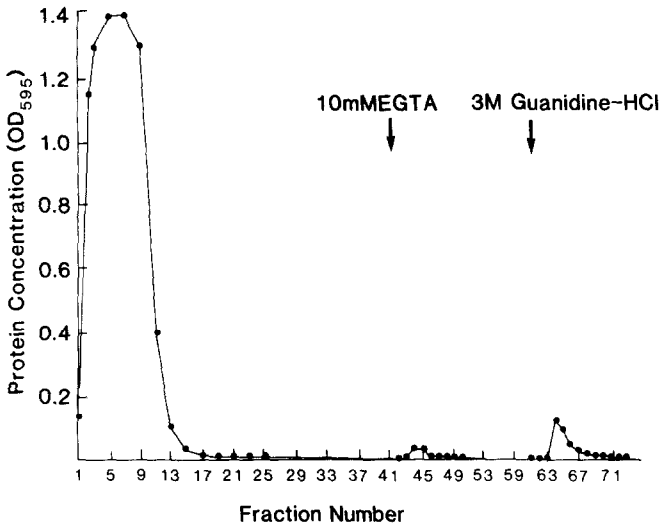

Figure 1 DNase I Sepharose affinity chromatogram.

The dialyzed Triton X-100 extract of thyroid plasma membranes was applied to a DNase I-sepharose affinity column. After thorough washing of the column with G buffer, proteins were eluted with 10 mM EGTA in G buffer followed by 3 M guanidine-HCl. Protein concentration was determined by the method of Bradford (25). Sample fractions were 0.8 ml.

was applied to a DNase I Sepharose affinity column (1.5 cm x 6 cm). After the column was thoroughly washed with G buffer, protein was eluted with 10 mM EGTA in G buffer followed by 3 M guanidine-HCl (Figure 1). The 10 mM EGTA eluate was pooled, concentrated by collodion bag, dialyzed extensively against G buffer and stored at -70°C. The same protein peak could be eluted by increasing the NaCl concentration to 50 mM.

In SDS-gel electrophoresis the EGTA eluate revealed a single protein band of molecular weight approximately 42,000 (Figure 2, lane 4), differing in mobility from skeletal muscle actin lanes 4 and 6). The protein is designated as 42K protein. Inclusion of protease inhibitors such as phenylmethylsulfonyl fluroide, antipain and leupeptin during the purification produced identical results (data not shown). Thyroid actin was eluted with 3 M guanidine-HCl (Figure 2, lane 5).

The 42K protein markedly accelerated the initial phase of skeletal muscle actin polymerization in the presence of 30 mM KCl (Figure 3a). The effect was dose-dependent and was observed at substoichiometric amounts of protein. With

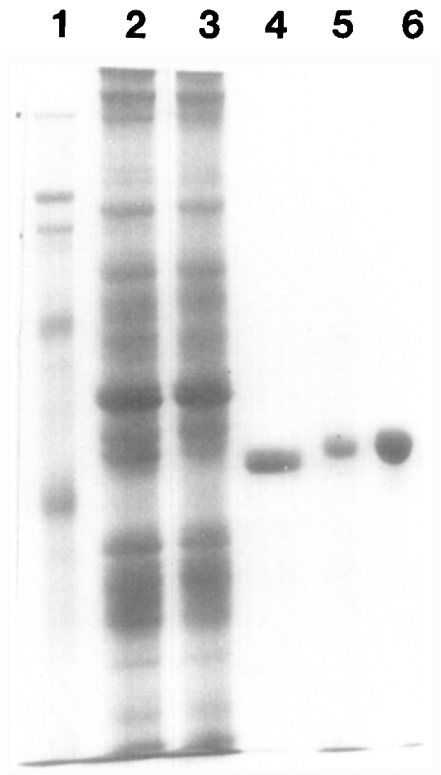

Figure 2 SDS-PAGE pattern at various steps of DNase I affinity chromatography.

1, molecular weight standard proteins, from the top, heavy chain of myosin (200,000), β-galactosidase (115,000), phosphorylase B (92,000), bovine serum albumin (68,000) and ovalbumin (45,000); 2, fraction before loading on the DNase column; 3, pass-through fraction from the DNase column; 4, 10 mM EGTA eluate; 5, 3 M guanidine-HCl eluate; 6, skeletal muscle actin.

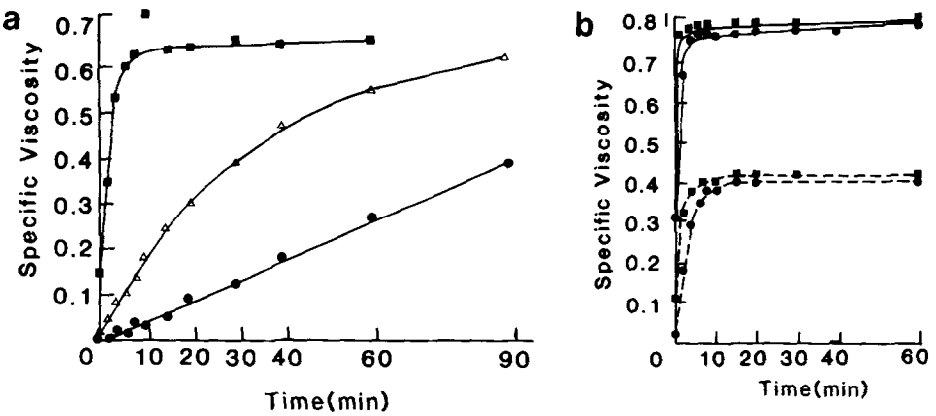

Figure 3 Effect of 42K protein on actin polymerization observed by Ostwald viscometry.

Panel a, 30 mM KCl; Panel b, 100 mM KCl and 2 mM $MgCl_2$.
●, without 42K protein; ■, with 6.3 ug/ml of 42K protein; ▲, with 3.15 ug/ml of 42K protein.
The actin concentration was either 600 ug/ml (————) or 300 ug/ml (- - - -).

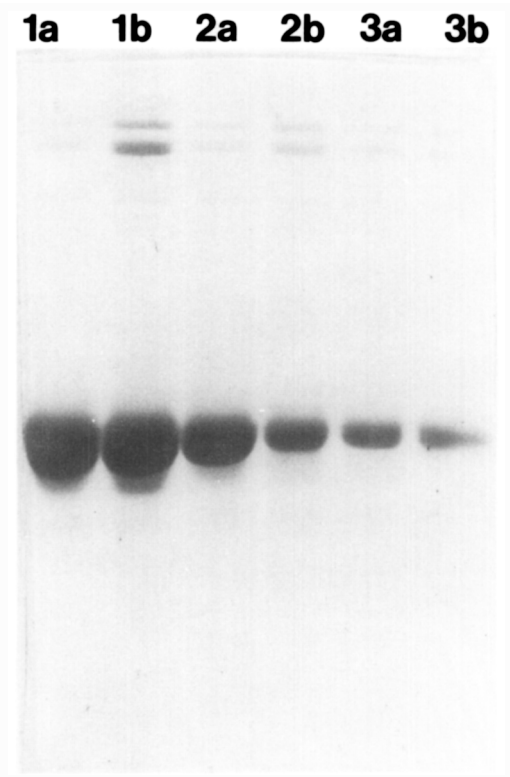

Figure 4 Effect of the 42K protein on actin polymerization analyzed by the centrifugation method.

Actin (60 ug) was incubated without (lanes a) or with (lanes b) the 42K protein (0.63 ug) in 100 ul final volume which contained: 1) no KCl; 2) 30 mM KCl; 3) 2 mM $MgCl_2$ and 100 mM KCl. After centrifugation, the same volumes of the supernates were subjected to SDS-gel electrophoresis.

100 mM KCl and 2 mM $MgCl_2$, the effect of the 42K protein was much less dramatic but could be increased by reducing the actin concentration by half (Figure 3b). Under these conditions, there was no significant effect of the 42K protein on the final viscosity (even after 24 hrs of incubation). The 42K protein did not affect actin polymerization in the absence of KCl.

Figure 4 demonstrates the effect of 42K protein on skeletal muscle actin polymerization as determined by the amount of G-actin remaining in the supernatant solution after centrifugation. Incubation prior to centrifugation was done without KCl, with 30 mM KCl and with 100 mM KCl and 2 mM $MgCl_2$. Without KCl this 42K protein had no significant effect on actin polymerization (lanes 1a and 1b). However, with 30 mM KCl, it significantly accelerated actin filament formation as indicated by the reduced amounts of G-actin remaining in the super-

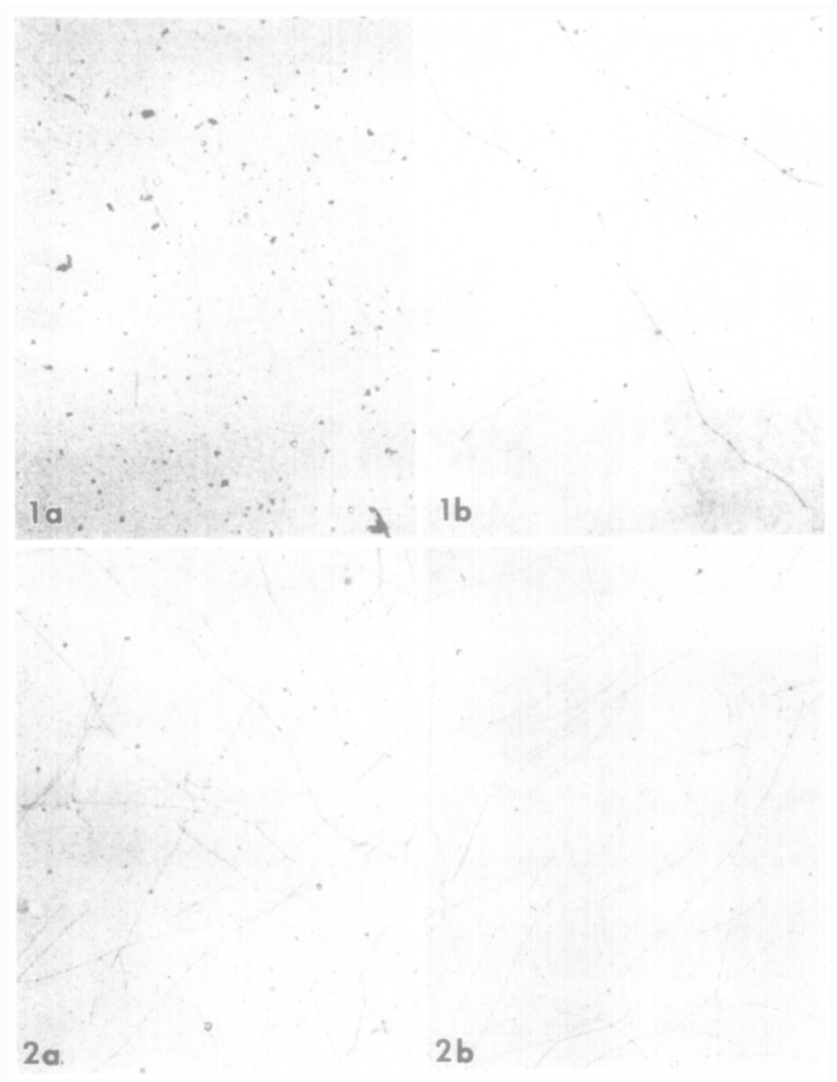

Figure 5 Electron microscopic appearance of actin filaments.

Actin (300 ug/ml) was incubated for 10 min at 25 C in the absence (panels 1a and 2a) or presence (panels 1b and 2b) of 42K protein (3.15 ug/ml). The incubation media contained either 30 mM KCl (panels 1a and 1b) or 2 mM $MgCl_2$ and 100 mM KCl (panels 2a and 2b). Magnification was 16,800 x.

natant (lanes 2a and 2b). With 100 mM KCl and 2 mM $MgCl_2$, actin polymerization was greatly facilitated and the effect of the 42K protein was less apparent (lanes 3a and 3b).

The 42K protein increased the length of actin filaments examined by electron microscopy (Figure 5, 1a and 1b). This effect was not clearly observed with 100 mM KCl and 2 mM $MgCl_2$ (2a and 2b).

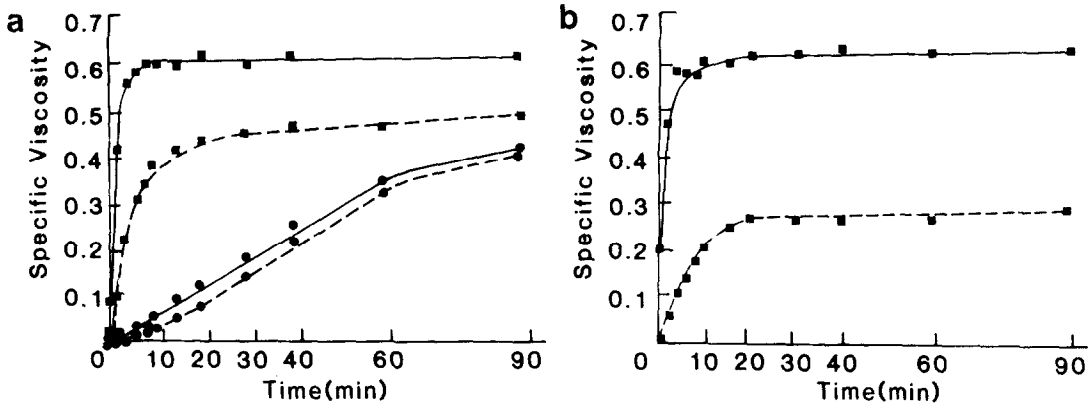

Figure 6 Effect of cytochalasins on actin polymerization.

Panel a is with 5 uM cytochalasin B; Panel b, 1 uM cytochalasin D. Actin (600 u/ml) was incubated without 42K protein (●), with 6.3 ug/ml of 42K protein (■), without cytochalasins (——), or with cytochalasins (- - - - -).

Cytochalasin B inhibited actin polymerization only slightly (Figure 6a), while in the presence of the 42K protein, it inhibited the acceleration of actin polymerization and the final viscosity. Figure 6b demonstrates similar results using 1 uM cytochalasin D.

Discussion

A 42,000 dalton stimulator of actin polymerization has been purified from bovine thyroid plasma membranes. Inclusion of protease inhibitors during the purification produced identical results, suggesting that this protein is not a degradation product of other proteins. It accelerated actin polymerization as demonstrated by its effect on the precipitation of F-actin, high shear viscometry and filament length observed by electron microscopy.

Several proteins which have biological effects on actin polymerization have been purified (8-16). All the nucleator proteins such as gelsolin (10), villin (11-13), fragmin (14), 90K protein (15) and 40K and 80K proteins (16) increase the initial (nucleation) phase of actin polymerization, but decrease the final viscosity. The 42K protein differs from these other proteins since it did not decrease the final viscosity. However, it may have a "seeding" effect on nucleation, thereby facilitating actin polymerization. The 42K protein from thyroid plasma membranes is quite similar in its effect on actin polymerization

to eu-actinin purified from skeletal muscle (25). By SDS-gel electrophoresis, the molecular weight of eu-actinin is similar to actin but eu-actinin forms dimers at low ionic strength. Eu-actinin facilitated the initial phase of actin polymerization and was localized by immunofluorescence on the Z-line of skeletal muscle from which actin filaments extend (25). Since 42K protein was purified from thyroid plasma membranes and has effects on actin polymerization similar to eu-actinin, it could be an anchoring site of actin filaments on the thyroid plasma membranes, important for generation of the mechanical force necessary for endocytosis. We have demonstrated an association of myosin and actin with thyroid plasma membranes (5). In order for these substances to generate any mechanical force or tension, one of them has to be attached to some relatively large structure such as the plasma membrane.

Cytochalasins affect various phenomena related to cell motility such as locomotion, cytoplasmic streaming, axonal growth, microfilament assembly, cytokinesis and endocytosis (26). Although the precise mechanism of such inhibition is not known, microfilament disruption has been implicated (26) since cytochalasins bind to the growing end of actin filaments (27-29). Inhibition of the effect of 42K protein by cytochalasins suggests that the binding site for cytochalasins and the 42K protein may be similar.

While thyroid gland has actin polymerization inhibitors (9,16) in addition to this 42K protein, the regulation of actin polymerization in situ is unknown. Maintenance of the integrity of the actin filaments by proteins that influence actin polymerization may be important for the interaction with myosin filaments.

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